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MINIREVIEW

Heme Synthesis in the Rhizobium-Legume Symbiosis: a Palette for Bacterial and Eukaryotic Pigments

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Rhizobia elicit nitrogen-fixing nodules on legume roots composed of highly differentiated plant and bacterial cells. Sustaining symbiosis is energy intensive, and an increase in plant and bacterial heme protein synthesis is an essential feature of nodule ontogeny. Symbiotic bacteroids express cytochrome heme proteins as part of an electron transport system that allows vigorous and efficient respiration in the low-O_2 milieu of nodules to support nitrogen fixation. Bacteroid respiration is also dependent on leghemoglobin, an abundant plant protein in nodules that facilitates O_2 diffusion to the endosymbiont. Interest in heme synthesis in the rhizobium-legume symbiosis has been stimulated by speculation that the heme prosthetic group of leghemoglobin is synthesized by the bacterial symbiont. As will be discussed, recent work suggests that this hypothesis is incorrect, but the idea has inspired experiments that have uncovered novel mechanisms for heme synthesis in nodules. These studies indicate that the seemingly esoteric processes involved in symbiotic development are variants of themes found in other prokaryotes and eukaryotes. Because they accent these themes, the symbiotic systems are excellent models for their study and have contributed to the refinement of paradigms that integrate these processes into the framework of biology.

BACKGROUND ON RHIZOBIUM-LEGUME SYMBIOSES

Bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, collectively referred to as rhizobia, form symbioses with leguminous plants. In general, a rhizobial species interacts with one or a limited number of plants, and the molecular basis of this specificity has been elucidated by a spectacular body of work from several groups (reviewed in references 24 and 27). Symbiotic rhizobia resemble an organelle in that they are an intracellular structure with a specialized role in the maintenance of the eukaryote, and 16S rRNA analysis shows that rhizobia and numerous other bacteria that form close associations with eukaryotes are related to mitochondria (95). Bacteroids reduce atmospheric nitrogen to ammonia at the expense of 16 to 42 ATP molecules per N_2-molecule, and the fixed nitrogen is assimilated by the plant host. Cytochrome heme protein content increases during bacteroid development to accommodate this high energy demand, and an altered electron transport pathway is expressed that includes a cytochrome oxidase that can function in the low-O_2 environment of a nodule (49, 67). Heme types *a*, *b*, *c*, and *d* are all represented in the rhizobia, although not necessarily in each species or under every growth condition. Virtually nothing is known about the covalent modifications of protoheme for synthesis of *a*- and *d*-type heme in rhizobia, and recent findings for *c*-type heme are described below. In addition to the cytochrome heme proteins, FixL is a heme-containing histidine kinase that senses and binds O_2, and the deoxygenated protein initiates a signal transduction pathway leading to the expression of genes needed for bacteroid development (33).

The plant host is an active participant in bacteroid respiration via leghemoglobin, which facilitates O_2 diffusion to the endosymbiont. Plant hemoglobin is essential to nitrogen-fixing symbioses, and the concentration must be high in order for it to facilitate the diffusion of a molecule much smaller than itself (see reference 3). Indeed, the leghemoglobin concentration within the cytoplasm of infected nodule cells is about 3 mM, necessitating a high level of tetrapyrrole synthesis for nodule formation. Once thought to be confined to legume nodules, hemoglobins are very likely to be dispersed throughout the plant kingdom. The function of these asymbiotic hemoglobins is unknown, but roles in O_2 transport or sensing have been suggested (4).

HEME SYNTHESIS IN BRIEF

Hemes are ubiquitous in living organisms, and heme proteins are directly involved in many reactions that require oxidation-reduction, oxygenation, hydroxylation, and binding of oxygen and other diatomic gases. In addition, heme proteins are now known to be sensors in O_2- and nitric oxide-dependent signal transduction pathways in rhizobia (33) and in animals (35, 55, 90). Protoheme is synthesized from the universal tetrapyrrole precursor δ-aminolevulinic acid (ALA) by seven successive enzymatic reactions with well-described intermediates (reviewed in references 20 and 46; Fig. 1). Uroporphyrinogen III, an intermediate in the pathway, is a precursor for vitamin B_{12}, siroheme, and other tetrapyrrole derivatives in organisms that express them. Chlorophyll and heme synthesis diverge at protoporphyrin IX, and plant hemes are quantitatively minor tetrapyrroles in photosynthetic tissues where chlorophyll is abundant. Hemoglobins, cytochromes P-450, catalase, peroxidase, and *b*-type cytochromes all have protoheme as the prosthetic group; protoheme is modified further to synthesize *a*-, *c*- and *d*-type cytochrome hemes, and the mesoheme moiety of cytochromes *c* is covalently attached to the protein.

ALA synthase and the C_5 pathway are the two known routes of ALA formation (reviewed in references 9 and 43). ALA synthase is found in animals, yeasts, and some bacteria. ALA formation by bacteria was originally thought to proceed exclusively by ALA synthase because of the historical precedent established by the animal studies and because of the pioneering biochemical and genetic work carried out with *Rhodobacter*

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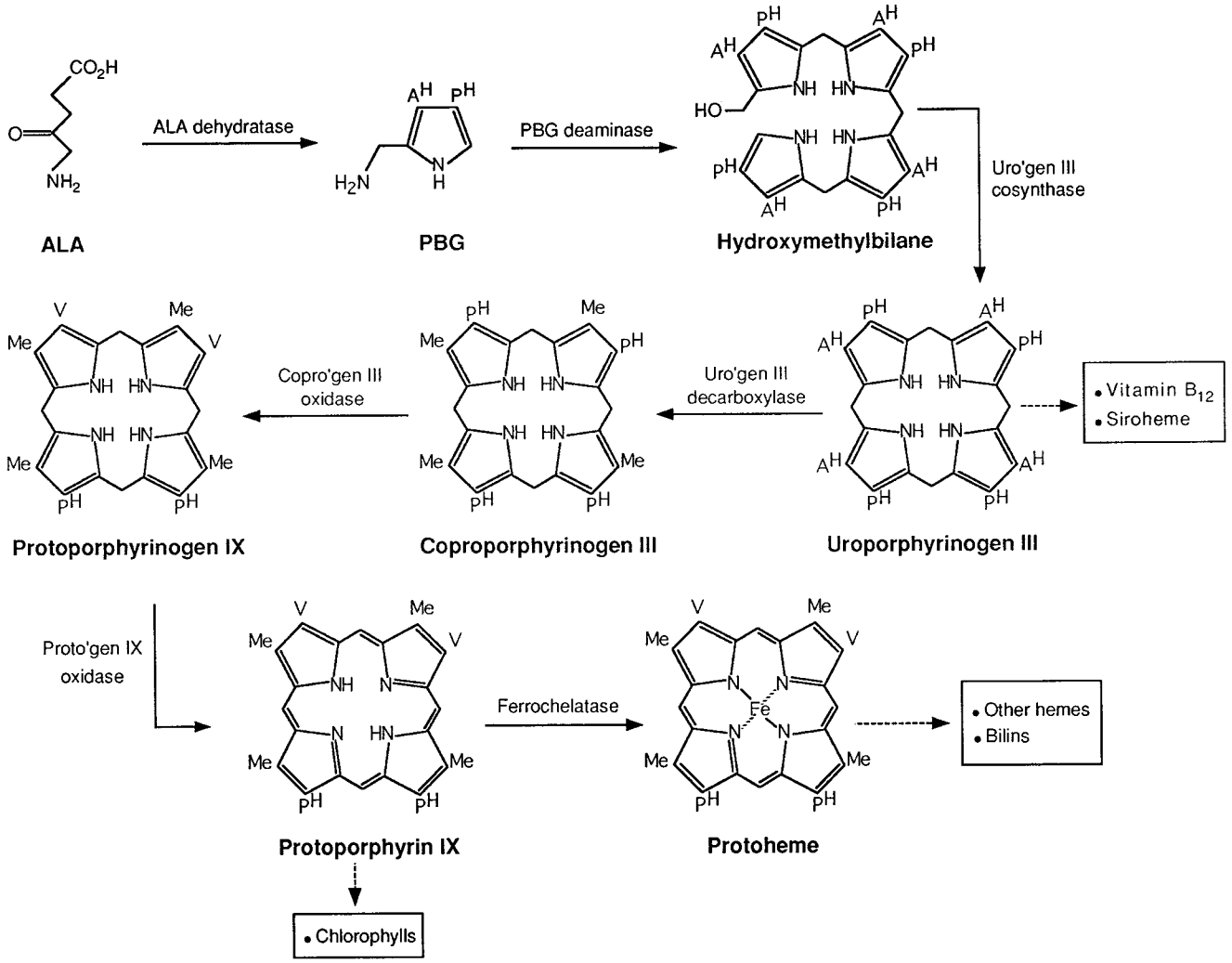

FIG. 1. Generic heme synthesis pathway from the universal tetrapyrrole precursor ALA. The dashed arrows indicate multistep reactions leading to other tetrapyrrole derivatives. In addition to substrates shown, coproporphyrinogen oxidase and protoporphyrinogen oxidase require O_2 in aerobic systems and another oxidant in anaerobic systems. Ferrochelatase requires ferrous iron. Abbreviations: ALA, δ-aminolevulinic acid; PBG, porphobilinogen; uro'gen, uroporphyrinogen; copro'gen, coproporphyrinogen; proto'gen, protoporphyrinogen; Me, methyl; A^H, acetyl; P^H, propionyl; V, vinyl.

sphaeroides (60) and *Rhizobium meliloti* (54), respectively. (This assumption led to the unfortunate and persistent "*hemA*" designation for the gene encoding either bacterial ALA synthase or the C_5 pathway enzyme glutamyl-tRNA reductase. These dissimilar proteins catalyze different reactions, and *hemA* will refer to the ALA synthase gene in this review.) It now appears that the C_5 pathway is prevalent in bacteria, and ALA synthase is confined to the α-subgroup of purple eubacteria (7), to which the genera *Rhizobium* and *Rhodobacter* belong. The putative endosymbiotic bacterial ancestor of mitochondria belongs to this subgroup as well (95), and eukaryotic ALA synthase is localized to that organelle in organisms that express it. ALA synthesis from glutamate by the C_5 pathway is a three-step process and involves a tRNA in an unusual context (Fig. 2). Plants and algae express the C_5 pathway, and this system was originally thought to be dedicated to ALA destined for chlorophyll. However, the C_5 pathway is probably involved in synthesis of all plant tetrapyrroles, and convincing evidence for an ALA synthase is lacking (9). This view is supported by the presence of the C_5 pathway, but not ALA synthase, in root nodules, an organ which contains heme but not chlorophyll (see below).

Heme pathway enzymes are spatially separated within eukaryotic cells (20, 78, 79), and this concept is important to this minireview, as described below. An obvious implication of this organization is that heme precursors must be transported from one space to another for synthesis of the end product. In animals, ALA synthase is found in the mitochondrial matrix, and the final three enzymes, coproporphyrinogen oxidase, protoporphyrinogen oxidase, and ferrochelatase, are associated with the inner mitochondrial membrane. However, the enzymes catalyzing the intermediate steps are cytosolic. Plant plastids contain all the enzymes for chlorophyll and heme synthesis, but plant mitochondria contain only protoporphyrinogen oxidase and ferrochelatase. Thus, mitochondrial heme formation requires a precursor formed in plastids.

LEGHEMOGLOBIN HEME

Leghemoglobin is the predominant plant protein in nodules, and induction of synthesis is concomitant with that of nitrogen fixation during nodule development. Leghemoglobin facilitates the diffusion of oxygen to nodule bacteroids to support nitrogen fixation, and for many years plant hemoglobin was thought

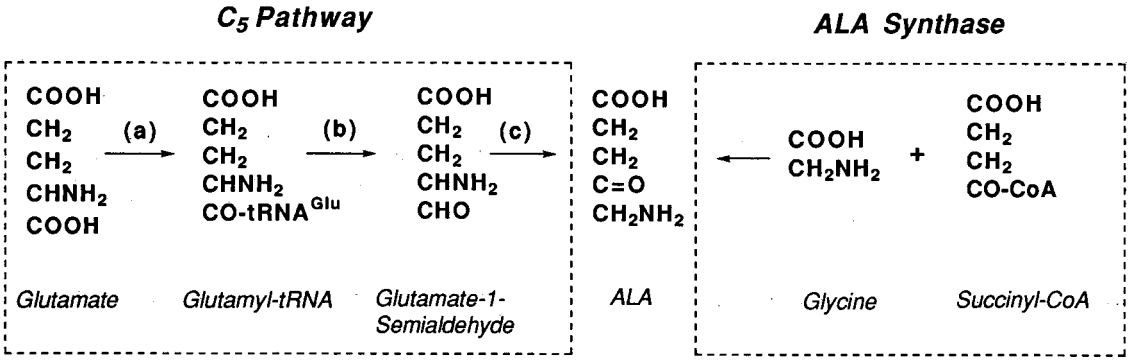

FIG. 2. ALA synthesis by ALA synthase or the C_5 pathway. The enzymes of the C_5 pathway and their cofactors are as follows: (a) glutamyl-tRNA synthesis (requires Mg-ATP); (b) glutamyl-tRNA reductase [pyridoxal phosphate dependent and requires NAD(P)H]; and (c) glutamate 1-semialdehyde aminotransferase.

to be unique to leguminous symbioses. However, functional hemoglobin genes have been found subsequently in nodules of the symbiotic nonlegumes *Parasponia andersonii* (5) and *Casuarina glauca* (53) and also in the nonsymbiotic plants *Trema tomentosa* (13), barley, maize, wheat, and rye (84). In addition, hemoglobin genes distinct from nodule-specific ones are expressed in roots and leaves of *Casuarina glauca* (41) and soybean (1). The common evolutionary origin of plant and animal hemoglobins predicts that they are prevalent in the plant kingdom, and the identification of hemoglobins in asymbiotic contexts suggests that legume nodule hemoglobin is a specialized adaptation of a general plant process. The heme prosthetic group of asymbiotic hemoglobins must be synthesized entirely by the plant, and recent evidence shows that plant heme synthesis genes are strongly induced in legume nodules to levels that can support hemoglobin formation. From this, it seems that leghemoglobin heme is synthesized by the plant. However, the hypothesis that leghemoglobin heme is synthesized by the bacterial symbiont is a deeply entrenched idea, and is even cited matter-of-factly in textbooks (e.g., see reference 14). This hypothesis is founded on a report concluding that soybean cannot synthesize heme in nodules and that *Bradyrhizobium japonicum* makes and exports heme (19). Similar results were described for lupine nodules, but those authors were reluctant to conclude that hemoglobin heme is a bacterial product (34). Subsequently, it was reported that *B. japonicum* ALA synthase activity correlates with leghemoglobin accumulation in developing nodules (59). Finally, the absence of leghemoglobin in nodules elicited by some *Rhizobium* mutants was interpreted as corroboration of the bacterial origin hypothesis (61). A reassessment of the early work with hindsight provided by current information shows that these conclusions are not correct, and therefore the bacterial origin hypothesis does not withstand scrutiny. Each of these points is discussed in turn.

Plant heme synthesis in nodules. A deficiency in nodule heme synthesis activity by legumes, if true, would indeed be an indirect but compelling argument in favor of a bacterial source for the hemoglobin prosthetic group. Glutamate-dependent ALA synthesis by the C_5 pathway was not yet discovered when the early work on nodule heme synthesis was carried out (19, 34) and was initially thought to be limited to chlorophyll synthesis even after it was described. ALA synthesis by soybean and cowpea in nodules is now well documented (31, 58, 71–73) and is described in detail in the subsequent section. The C_5 pathway gene *Gsa1* is strongly induced in soybean nodules to levels similar to or greater than that found in leaves for chlorophyll synthesis (31, 73). Thus, plant ALA synthesis capacity in nodules is sufficient for hemoglobin heme formation. Also, glutamate is one of the most abundant compounds in nodules (83) and is unlikely to be a limiting factor in plant ALA formation. Contrary to previous reports, nodule ALA is metabolized by the plant host; soybean ALA dehydratase (48) and coproporphyrinogen oxidase (57) genes are strongly induced in nodules, and ferrochelatase activity has been measured in nodule mitochondria (26). The inability to demonstrate heme synthesis from radiolabeled ALA in the "plant" fraction of soybean or lupine nodules was erroneously interpreted because the organelle localization of heme pathway enzymes in plants was not yet known. Recall that while plastids contain the entire heme pathway, mitochondria harbor only the enzymes that catalyze the final two steps (78, 79). In labeling experiments, Cutting and Schulman (19) prepared nodule bacteroids devoid of mitochondria by disruption of nodules in an osmoticum followed by a centrifugation at 4,500 $\times$ *g*. However, all known types of undisrupted plant plastids will sediment under that force (47, 66, 77, 91) along with bacteroids, thus any heme formed from radiolabeled ALA by plant plastids would be incorrectly interpreted. The mitochondria remaining in the supernatant cannot synthesize heme directly from ALA due to an incomplete pathway, thus plant heme formation in either organelle would not have been observed.

Heme export by bacteroids. Heme export by *B. japonicum* bacteroids was discerned as the incorporation of radiolabel into extracellular heme from [^{14}C]ALA (19) and subsequent association with exogenous apoleghemoglobin. In those experiments, the rate of export was about 0.01% of the rate of hemoglobin heme accumulation in soybean nodules (based on data from reference 59), and the authors needed to use bacteroids from 10 g of nodules to observe ca. 2 pmol of heme exported in 7.5 h (19). It is difficult to ascribe physiological relevance to this negligible rate of heme efflux.

Relationship between bacterial ALA synthase activity and leghemoglobin accumulation. A parallel increase in *B. japonicum* ALA synthase activity with hemoglobin accumulation as a function of soybean nodule age has been interpreted as an argument for a bacterial source for hemoglobin heme (59). However, the apparent increase in ALA synthase activity with time is only observed when the data are presented on a nodule fresh weight basis and can be attributed to the proportional increase in bacterial mass as a function of nodule age (72). When calculated as specific activity, ALA synthase activity remains constant in nodules and is similar to that observed in cultured cells (72). Thus, the results obtained for ALA synthase are expected for any constitutive bacterial activity in nodules. The linear accumulation of hemoglobin heme as a function of nodule age (59, 72) does not indicate a constant

increase in the rate of ALA synthesis but is consistent with an induction of the limiting step(s) followed by maintenance of the elevated rate. Whereas no induction of *B. japonicum* ALA synthase can be observed, soybean ALA synthesis is strongly induced and maintained in nodules (31, 72, 73).

Analysis of rhizobial heme mutants. The initial interest in rhizobial heme mutants was to assess the bacterial role in leghemoglobin heme synthesis, but evidence shows that these mutants do not address that issue. ALA synthase (*hemA*) mutants of *R. meliloti*, *Rhizobium* sp. NGR234, and *A. caulinodans* form undeveloped nodules on their respective hosts that do not fix nitrogen or express hemoglobin (54, 65, 82). However, heme is essential for viability and *hemA* mutants are ALA auxotrophs. Accordingly, ultrastructure analysis of alfalfa nodules elicited by an *R. meliloti hemA* mutant show that these nodules are arrested in early development and are devoid of intracellular bacteria (25). Leghemoglobin is normally expressed late in development, thus nodules arrested at an early stage will not express leghemoglobin regardless of the source of the heme prosthetic group. In the same study, leucine and adenosine auxotrophs showed the same phenotype as did the ALA auxotroph, thereby underscoring the conclusion that the leghemoglobin deficiency is a direct result of developmental arrest and not of heme synthesis per se. Similarly, inoculation of *S. rostrata* stems with an *A. caulinodans hemA* mutant induces only swelling of adventitious root sites, similar to what is observed with water treatment alone (65). Root nodules do form from the *hemA* mutant, but they are either very small or display irregular morphology (65). A *B. japonicum* mutant deficient in protoporphyrinogen oxidase activity differs somewhat from the other mutants in that it is not an auxotroph and it elicits nodules that contain some bacteria and hemoglobin apoprotein but no heme (61). The mutant is defective in a gene involved in cytochrome *c* biogenesis (68) and thus the relationship between the mutation and the protoporphyrinogen oxidase deficiency is not clear.

CONTROL OF PLANT HEME SYNTHESIS IN NODULES

ALA formation in nodules is a unique plant problem in that there is a high level of synthesis in nonphotosynthetic tissue and none of the product is incorporated into chlorophyll. Thus, nodules provide a system for studying heme synthesis in plants. In addition, induction of ALA synthesis is a response to interactions with a bacterium and should be controlled by factors related to symbiosis and nodule development rather than to photosynthesis. Despite these singular features, recent studies underscore the similarities in ALA formation between leaves and nodules, indicating common mechanisms of synthesis and control for different tetrapyrroles and for different tissues in plants.

ALA formation from glutamate is induced 25- to 50-fold in soybean nodules compared with in uninfected roots (72), and the activity is very likely catalyzed by the C_5 pathway (31, 73). The heme-containing nodule lacks chlorophyll, thus the presence of C_5 pathway enzymes and the absence of ALA synthase in that organ strongly support the argument that the C_5 pathway is the source of ALA for all plant tetrapyrroles. Induction of nodule ALA synthesis activity is due, at least in part, to activation of *Gsa*, the gene encoding glutamate 1-semialdehyde (GSA) aminotransferase (31, 73; Fig. 2). GSA aminotransferase mRNA, protein, and enzyme activity are very low in uninfected roots but are strongly expressed in nodules and in leaves for heme and chlorophyll synthesis, respectively (31, 73). This control differs from what is observed in *Arabidopsis thaliana*, where *Gsa* mRNA levels are similar between tissues (40). Strong *Gsa* expression temporally precedes that of leghemoglobin in developing nodules (31), which is consistent with a high glutamate-dependent ALA synthesis activity in young nodules, where the leghemoglobin content is low but discernible (72). It is plausible that an increased demand for plant ALA is needed prior to leghemoglobin synthesis for heme-dependent respiration associated with cell division or for bacterial heme formation, as described in the subsequent section.

Evidence for two different enzymes with glutamyl-tRNA reductase activity is given for two bacterial species (42, 69, 89), and although no similar situation has been reported for plants, separate ALA pools for heme and chlorophyll synthesis within chloroplasts have been proposed (39). However, the data argue against significant activity of an enzyme other than the well-described GSA aminotransferase for heme and chlorophyll formation in soybean (31), and any heterogeneity is likely to be due to the presence of a single type of enzyme in multiple compartments. A GSA aminotransferase gene, *Gsa1*, corresponds to the cloned nodule cDNA and appears to be one of two *Gsa* genes in the soybean genome (31). By using a gene-specific probe, it was shown that *Gsa1* mRNA accumulates to high levels in nodules and leaves but not in uninfected roots (31). *Gsa1* mRNA is also strongly expressed in leaves of dark-grown (etiolated) plantlets and is slightly stimulated by subsequent light exposure. The expression pattern of *Gsa1* mRNA is qualitatively similar to that of total *Gsa*, indicating either that the putative second *Gsa* gene is not expressed in the tissues examined or that its pattern of expression is similar to that of *Gsa1*. These data strongly suggest that *Gsa1* is a universal tetrapyrrole synthesis gene in soybean and that a *Gsa* gene specific for a tetrapyrrole, tissue, or light condition is unlikely. Thus, symbiosis with *B. japonicum* must alter the spatial expression of a soybean gene normally expressed strongly only in photosynthetic tissues for ALA synthesis in nodules. The activation of *Gsa1* in nodules also implies that strong ALA synthesis can be uncoupled from chloroplast development (10), hence *Gsa1* may be affected by separate and independent signal transduction pathways.

The *Gsa1* gene promoter contains an element found in numerous *Drosophila melanogaster* genes but which has not been found in other plant genes. The so-called GAGA element of *Gsa1* is a perfect dinucleotide repeat of $(dG\text{-}dA)_9 \cdot (dT\text{-}dC)_9$, and this DNA has single-stranded character in vitro as discerned by S1 nuclease sensitivity (31). The GAGA element forms a complex with nuclear factors from nodules and leaves but not with those from uninfected roots, and thus complex formation correlates with activation of *Gsa1* transcription. In *D. melanogaster*, GAGA elements are involved in chromatin remodeling; binding of the DNA to a GAGA-binding factor disrupts nucleosomes locally and renders the promoter accessible to RNA polymerase and transcription factors (56, 80, 87). There are now numerous other examples of involvement of simple repeat DNA in chromatin structure and gene regulation in animals (76), and it will be interesting to learn the role it plays in plants and in nodule development and function.

ALA is immediately metabolized by ALA dehydratase to form porphobilinogen (Fig. 1), and the gene or cDNA encoding ALA dehydratase (*Alad*) has been cloned from pea (12) and soybean (48) and from numerous non-leguminous plants as well (48). Like GSA aminotransferase, soybean ALA dehydratase is strongly induced in nodules, and little protein or enzyme activity is found in uninfected roots (48). *Alad* expression appears to be controlled at protein synthesis or turnover in nodules rather than at transcription, as message levels are relatively high in roots despite the absence of detectable protein. The soybean gene encoding coproporphyrinogen oxidase

(*Cpox*), the enzyme which catalyzes the antepenultimate step in heme synthesis (Fig. 1), has been obtained as well, and it was initially identified by its abundant message in nodules (57). Induction of *Cpox* mRNA is temporally comparable to that of leghemoglobin; its induction relative to *Gsa1* has not been assessed. There appears to be only one copy of *Cpox* in the soybean genome and neither it nor *Gsa1* have sequences in their promoters with homology to elements involved in nodule-specific expression. Thus, like *Gsa1*, *Cpox* expression in soybean nodules probably does not involve induction of an organ-specific subset of a gene family but rather the altered expression of a gene not normally expressed strongly in root-derived tissue.

RHIZOBIAL HEME MUTANTS AND THE *hemA* PARADOX

Although rhizobial heme mutants do not address the source of leghemoglobin heme as explained above, their utility in studying bacterial heme formation has been significant. ALA synthesis in rhizobia occurs by ALA synthase in all species where it has been examined (7, 54, 59, 65, 82), and the gene encoding it (*hemA*) has been cloned in each case (36, 54, 65, 82). Activity of the C_5 pathway could not be demonstrated in *Azorhizobium caulinodans* (7) or *B. japonicum* (30, 71), and its absence can be inferred in cultured cells of other rhizobia by the ALA auxotroph phenotype of *hemA* mutants (54, 82). However, interesting pseudorevertants of an *A. caulinodans hemA* mutant have been isolated that grow in complex media, but not in minimal media, in the absence of additional ALA; hence, those cells may express C_5 pathway enzymes (65). If so, the activity is likely to be low because, like the *hemA* strain, the double mutant is not a true ALA prototroph, it could not establish effective symbiosis with *Sesbania rostrata*, and C_5 pathway activity was undetectable (65). These pseudorevertants are defective in free-living nitrogen fixation and assimilation and hint at a potentially exciting interrelationship between nitrogen and heme metabolism.

R. meliloti (54) and *A. caulinodans* (65) *hemA* mutants form undeveloped nodules on alfalfa and *S. rostrata*, respectively, that do not fix N_2 or contain leghemoglobin. Similarly, a *hemA* mutant of broad host range bacterium *Rhizobium* sp. NGR234 elicits ineffective nodules on numerous legume hosts (82). However, a *hemA B. japonicum* strain forms effective nodules on soybean, showing that ALA synthase is not essential for that symbiosis (36). This latter finding is remarkable because heme is required for cell viability and proliferation of an aerobic bacterium and also because it contrasts with the other rhizobia examined. In addition, *B. japonicum hemB* (15) and *hemH* (28, 29) mutants do not effectively nodulate soybean, and thus the problem is specific to *hemA* in that bacterium rather than to the pathway in general. Data indicate that the *B. japonicum hemA* mutant is rescued symbiotically on soybean by provision of plant-derived ALA to the bacterial endosymbiont (71). Whereas heme is not detected in cultured cells of the *hemA* mutant, it is expressed in nodules despite the absence of bacterial ALA synthase or C_5 pathway activities (30, 71). In addition, *B. japonicum* bacteroids take up exogenous ALA, and the rate of uptake is comparable to that observed for other organic acids essential for bacterial metabolism (58, 71). The presence of the heme precursor porphobilinogen in the *hemA* mutant (74) and the requirement of *hemB* for symbiosis (15) indicate that ALA is the only precursor that can be translocated to support bacterial heme synthesis.

The difference between the *B. japonicum*-soybean symbiosis and the other systems with regard to the bacterial *hemA* requirement can apparently be reconciled by the ALA rescue hypothesis as well. A *B. japonicum hemA* mutant forms nitrogen-fixing nodules on several legume hosts, including cowpea (58), a plant that is not effectively nodulated by a *hemA* mutant of *Rhizobium* sp. strain NGR234 (82). Cowpea synthesizes ALA in nodules elicited by either *B. japonicum* or *Rhizobium* sp. strain ANU240 (a streptomycin-resistant derivative of strain NGR234), but only the *hemA* strain of *B. japonicum* is successful on that host (58). Finally, the ALA uptake activity observed in *B. japonicum* is severely deficient in species of *Rhizobium* that require *hemA* for symbiosis (58). Collectively, the data suggest that the legume hosts, at least soybean and cowpea, synthesize ALA in nodules, but only *B. japonicum* can utilize that ALA due to an uptake activity. It is not yet known whether plant ALA normally contributes to *B. japonicum* heme formation in nodules, or only when the endosymbiont is impaired in ALA synthesis.

Symbiotic bacteroids are surrounded by a peribacteroid membrane of plant origin to form a structure termed a symbiosome. Assuming that ALA is synthesized in plastids, or at least outside of the symbiosome, then ALA would have to traverse the peribacteroid membrane to be metabolized by bacteroids. Herrada et al. reported a low ALA uptake activity for French bean symbiosomes and stated, without presenting evidence, that soybean symbiosomes were quantitatively the same (37). However, the ALA uptake activity shown in that study was severalfold higher than the rate at which ALA is subsequently consumed by the *B. japonicum* ALA dehydratase (71). Therefore, the data do not demonstrate that ALA uptake across the peribacteroid membrane would be the limiting step of bacteroid heme synthesis. It should be kept in mind that the influx of a committed precursor to a stable molecule such as heme need not be as great as the uptake of dicarboxylates (88) that serve as carbon and energy sources and which are rapidly consumed.

ROLE OF OXYGEN IN RHIZOBIAL HEME SYNTHESIS

The nodule is a microaerobic environment, and oxygen is a key regulator of symbiotic functions in rhizobia (81 and references therein). Unlike many bacterial components important for nitrogen fixation, hemes are expressed under all cellular conditions, and a 10-fold stimulation in heme expression by restricted aeration has been reported for *B. japonicum* cultured cells (6). In addition, heme synthesis involves two oxidation steps catalyzed by coproporphyrinogen oxidase and protoporphyrinogen oxidase, which use O_2 in aerobically grown cells and which must be accounted for under oxygen-limiting conditions. *B. japonicum* has an O_2-independent coproporphyrinogen oxidase activity that is present in cells grown anaerobically, but not in those grown aerobically (50). This work has not been pursued further; further study might be fruitful, as this is likely to be a regulated step important to symbiosis. It is now known that several bacteria have both aerobic and anaerobic coproporphyrinogen oxidases encoded by different genes (18, 32, 86, 92–94), which could explain the findings for *B. japonicum.*

ALA synthase and ALA dehydratase activities are induced under O_2 deprivation in *B. japonicum* cultured cells, and this induction correlates with that of heme in those cells (6). In addition, the *hemA* promoters from *B. japonicum* and *R. meliloti* contain a DNA region with significant homology to the DNA binding site of *E. coli* Fnr (22, 62). Fnr is a transcriptional regulator that mediates the control of numerous *E. coli* genes by oxygen, and an Fnr homolog, called FixK, has been identified that is indeed a regulator of O_2-dependent transcrip-

tion of some rhizobial genes (2, 8, 17). In a study using a *hemA-lacZ* fusion, deletion of a putative Fnr (FixK?) binding site results in loss of O_2-dependent induction of B-galactosidase activity in *B. japonicum* cultured cells (63). Relating work with *B. japonicum* cultured cells to symbiosis is tempered somewhat by the fact that is not obvious that *hemA* induction is necessary in nodules; ALA synthase activity is no greater in nodules than it is in aerobic cultured cells (72), and a *hemA* mutant completely defective in ALA synthase expresses 60 and 90% of wild-type levels of cytochrome heme in soybean and cowpea nodules, respectively (58). The apparent constitutive activity in nodules may be a composite of both activation and repression, as has been observed for ALA synthase in *Saccharomyces cerevisiae* (51). In this case, the O_2-limited cultures would simulate an inducing environment in the absence of repressing conditions, and predicts that loss of O_2 control in nodules would repress *hemA* expression. Induction of ALA synthesis in response to O_2 limitation is not unique to symbiotic bacteria (21), and control of *hemA* may be relevant to free-living rhizobia in soil.

ALA DEHYDRATASE IN RHIZOBIA AND IMPLICATIONS FOR DEHYDRATASES IN PLANTS AND ANIMALS

ALA dehydratase catalyzes porphobilinogen formation from ALA (Fig. 2) and is encoded by *hemB* in bacteria. Unlike *hemA*, *B. japonicum hemB* is required for symbiosis with soybean and thus ALA dehydratase appears to catalyze the first essential bacterial step for *B. japonicum* heme synthesis in nodules. ALA dehydratase is a zinc-dependent enzyme in animals, yeasts, and some bacteria (reviewed in reference 45). Cysteine residues participate in Zn^{2+} binding, and these enzymes contain a cysteine-rich domain that may be involved. Plant dehydratases are localized to plastids and are needed for chlorophyll synthesis in addition to other cellular tetrapyrroles. They share 35 to 50% identity with the nonplant enzymes, but activity requires magnesium rather than Zn^{2+}. The peptide region in the plant enzyme that corresponds to the putative Zn^{2+} domain of animals lacks the cysteines and histidine residues, and contains aspartate, alanine, or threonine instead (12). *B. japonicum* ALA dehydratase is unusual in that it contains a Mg^{2+}-dependent enzyme even though it is nonphotosynthetic, and it contains a putative metal-binding domain that has some residues otherwise found in plants (15). A modified *B. japonicum* ALA dehydratase was constructed by site-directed mutagenesis of *hemB* in which three proximal amino acids conserved in plant dehydratases were changed to cysteine residues as is found in the Zn^{2+}-dependent enzyme of animals (16). These substitutions result in an enzyme that requires Zn^{2+} rather than Mg^{2+} for catalytic activity; therefore, a region of ALA dehydratase from *B. japonicum*, and probably from plants, was identified that is involved in Mg^{2+} dependence. Moreover, a change in only a few amino acids is sufficient to change an Mg^{2+}-dependent ALA dehydratase to a Zn^{2+}-dependent one. The presence of the Zn^{2+}-dependent dehydratase in eubacteria, archaebacteria, animals, and fungi suggests that it predates the Mg^{2+}-type enzyme, and the mutagenesis study implies that the Mg^{2+} enzyme could have arisen from the Zn^{2+} enzyme by minor alterations during evolution. It is not clear whether there is a physiological reason for ALA dehydratase with different metal requirements. A *B. japonicum* strain harboring the altered *hemB* in the genome elicits nitrogen-fixing nodules on soybean and expresses nearly wild-type levels of cytochrome heme (16). Thus, the plant milieu in nodules permits the functioning of a Zn^{2+}-dependent ALA dehydratase within the endosymbiont that supports tetrapyrrole synthesis. Whether such an enzyme would function in chloroplasts remains to be addressed.

CYTOCHROME *c* BIOGENESIS IN RHIZOBIA: A PARADIGM FOR BACTERIA AND ORGANELLES

Cytochromes *c* are found in many bacteria and in mitochondria and plastids of eukaryotes. The cytochrome *c* heme group is unique because it is covalently attached to the peptide by two thioether linkages between protein cysteines and the protoheme vinyl groups. Cytochrome *c* heme lyase catalyzes the heme attachment in eukaryotes, but almost nothing was known about maturation of *c*-type cytochromes until gene clusters that are homologous to each other were identified from *B. japonicum* (68) and *Rhodobacter capsulatus* (11). The first surprise is the number of essential genes common to synthesis of all *c*-type cytochromes in each organism, with seven genes localized in two clusters in *R. capsulatus* and nine *B. japonicum* genes also found in two clusters. Mutations in any of these genes results in the loss of all *c*-type cytochromes. The *cycV* and *cycW* genes from *B. japonicum* and the *helA* and *helB* genes from *R. capsulatus* are homologous to each other, respectively, and to those encoding members of the ABC (ATP-binding cassette) membrane transport superfamily described from other organisms. It was suggested that these proteins transport heme to the periplasm, where bacterial cytochrome *c* holoproteins reside. *tlpB* and *helX* from *B. japonicum* and *R. capsulatus*, respectively, encode periplasmic thioredoxin-like proteins that may be involved in reduction of apoprotein cysteine or heme iron for covalent attachment. Finally, *cycHJKL* genes from *B. japonicum*, as well as from *R. meliloti* and *R. leguminosarum*, might encode a heme lyase complex, but this complex has no homology to the eukaryotic lyases (23, 52, 70). Homologs of these *B. japonicum* and *R. capsulatus* genes have been subsequently identified in several bacteria, including *E. coli* (64, 85). Moreover, these genes have homologs in plant chloroplast genomes (11, 38) and in mitochondrial genomes of plants and a paramecium (11, 44, 74, 75), suggesting that cytochrome *c* biogenesis in some eukaryotes is complex and involves genes in addition to those known to be in the nucleus. The work in *B. japonicum* and *R. capsulatus* opens up exciting possibilities for those interested in organelle function, regulation, and the coordination of nuclear and organelle gene expression. It should be noted that the functions of the identified bacterial genes are tentative, and therefore the organellar homologs should be discussed with equal reserve.

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